DEVELOPMENTAL ANATOMY AND MORPHOLOGY OF THE FLOWERS AND FRUITS OF SPECIES FROM *GALIUM* AND *RELBUNIUM* (RUBIEAE, RUBIACEAE)[1]

Karen L. G. De Toni[2] *and Jorge E. A. Mariath*[3]

Abstract

The circumscription of genera within the family Rubiaceae is by no means a precise endeavor at the current time. For example, the genus *Relbunium* (Endl.) Hook. f. is currently included as a section of *Galium* L., sensu Dempster, although this placement does not have unanimous support among specialists. To clarify these uncertainties, this study examined whether *Relbunium* is an autonomous genus or a section of *Galium* by analyzing the morphology and development of flowers and fruits of the following species: *G. latoramosum* Clos, *G. uruguayense* Bacigalupo, *R. equisetoides* (Cham. & Schltdl.) Ehrend., *R. gracillimum* Ehrend., *R. hirtum* (Lam.) K. Schum., *R. humile* (Cham. & Schltdl.) K. Schum., *R. humilioides* M. L. Porto & Ehrend., *R. hypocarpium* (L.) Hemsl., *R. longipedunculatum* Mariath & Ehrend., *R. mazocarpum* Greenm., *R. megapotamicum* (Spreng.) Ehrend., *R. nigro-ramosum* Ehrend., *R. ostenianum* Standl., *R. richardianum* (Gillies ex Hook. & Arn.) Hicken, and *R. valantioides* (Cham. & Schltdl.) K. Schum. This morphological data set and analysis were then used to construct a cladogram, which indicates that *Galium* is a sister group to *Relbunium* and further characterizes *Relbunium* as a monophyletic clade that should be considered an autonomous genus.

Key words: Evolution, *Galium*, pericarp, phylogeny, *Relbunium*, Rubiaceae, seed, seed coat.

The family Rubiaceae has a cosmopolitan distribution and is frequently encountered in tropical and subtropical regions (Dillenburg, 1978). Members of this family can be easily recognized by the presence of an inferior ovary, opposite and decussate leaves with interpetiolar stipules with colleters, a nuclear endosperm, the widespread occurrence of alkaloids, and the absence of an internal phloem (Bremer, 1992, 1996). Despite the well-defined morphological characteristics that distinguish Rubiaceae from other plant families, intrafamily delimitations are complex. Moreover, the species of Rubiaceae are biologically and morphologically quite diverse and have distinct habits and reproductive characteristics (Bremer et al., 1995).

The parallel evolution of similar morphological characters within otherwise distinct groups, together with a scarcity of morphological and phylogenetic studies (Bremer & Jansen, 1991), have led to the lack of taxonomic consensus. This confusion relates to subfamilies and tribes, but there are also ongoing discussions concerning the delimitation of the component genera and species, such as the *Hedyotis* L.–*Oldenlandia* L. complex in the genus *Psychotria* L. (Bremer & Jansen, 1991) and the genera *Galium* L. and *Relbunium* (Endl.) Hook. f. (Dempster, 1981, 1982, 1990).

The first taxonomic studies of the genus *Relbunium* were published in 1839 and included 20 species for *Rubia* L. sect. *Involucratae* DC. (de Candolle, 1839), which was characterized by the presence of an involucre below the sessile flower. The name *Relbunium* was first proposed by Endlicher (1839) as a section within the genus *Galium*. Later, in 1873, a generic rank was recognized by Bentham and Hooker. Schumann (1889) cited 22 species for the genus *Relbunium* and established the appropriate binomials for the first time. In 1955, Ehrendorfer published a detailed description of *Relbunium*, recognizing 30 species distributed among sections,

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[2] Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Rua Pacheco Leão 915, Jardim Botânico, 22460-030, Rio de Janeiro, Rio de Janeiro, Brazil. karen@jbrj.gov.br.

[3] Laboratório de Anatomia Vegetal, Depto. Botânica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, Prédio 43423, Sala 206, 91540-000, Porto Alegre, Rio Grande do Sul, Brazil.

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subsections, and series. According to this author, the delimitations of the genera *Relbunium* and *Rubia* principally result from their respective geographic distributions, as well as differences in their growth forms, corolla, and fruit. Ehrendorfer (1955) suggested that the closer morphological relationships between the American species of *Galium* and *Relbunium* indicated that *Relbunium* originated from *Galium* in the New World. Dempster (1978) viewed *Relbunium* as a clearly monophyletic group, even though its species are often confused with those of *Galium*. This author further affirmed that the species of *Relbunium* are easily distinguished when flowering or fruiting, observing that involucres are absent in *Galium*, whereas species in *Relbunium* exhibit four involucral bracts subtending each flower. However, according to Dempster (1978), numerous other vegetative morphological characteristics are common to both genera, supporting *Relbunium* as a section of *Galium*, as first proposed by Endlicher (1839).

In 1982, Dempster mentioned that the genus *Relbunium* should be limited to taxa with solitary and essentially sessile flowers with involucres. Based on this observation, the author transferred all of the species without these characteristics into the genus *Galium*. In her last work with South American *Galium*, Dempster (1990) returned to the proposals of Endlicher (1839) and defined *Relbunium* as a section of *Galium*.

Plastid DNA sequence analyses of species of Rubieae (Natali et al., 1996) included *Relbunium* as *R. hypocarpium* (L.) Hemsl., but did not further address taxonomic issues. Bremer and Manen (2000) included 14 genera in their classification for the Rubieae and treated *Relbunium* separately from *Galium*.

Galium comprises approximately 400 species (Judd et al., 1999), of which ca. 90 are found in the Neotropics (Andersson, 1992). The genus *Relbunium* comprises ca. 35 Neotropical species that are distributed from the southwestern United States (Porto et al., 1977). These species preferentially grow in open fields and forest borders in subtropical regions. Approximately 22 species are known for the southern regions of South America, including southern Brazil, Uruguay, and Argentina (Chamisso & Schlechtendal, 1828; de Candolle, 1839; Schumann, 1891; Ehrendorfer, 1955; Smith & Down, 1956; Rambo, 1962; Porto et al., 1977). Approximately 39% of *Relbunium* species, representing over half of the described South American species of *Relbunium*, are included in the morphological survey herein.

Fruits and seeds are always important in taxonomic classifications of Rubiaceae, as can be seen in Schumann (1891), Bremekamp (1966), and Robbrecht (1988). In addition to characterizing subfamilies and tribes, fruits and seeds have been used to distinguish genera, as in the case of *Appunia* Hook. f. and *Morinda* L. (Hayden & Dwyer, 1969). These infrafamilial morphological variations indicate a diversity of adaptations, especially in terms of seed dispersal (Bremer & Eriksson, 1992). Even though previous studies have variously treated *Galium* and *Relbunium*, there has been insufficient work focusing on their taxonomy that could integrate the anatomical, morphological, ecological, and molecular evidences. Few works describe the morphology and anatomy of the flowers, fruits, and seeds of these taxa in terms of their phylogenetic significance. According to Corner (1976), there is a need for extensive ontogenetic investigations of the pericarp, integument thickness, and the structure of the seed coat at the genus and species levels in the Rubiaceae. Consequently, the present study examines the structure and development of the flowers, fruits, and seeds within exemplar species of *Galium* and *Relbunium* to elucidate additional taxonomic characteristics toward the delimitation and relative ranks of these two groups/taxa.

Materials and Methods

We examined 13 species of *Relbunium* (sensu Ehrendorfer, 1955, modified by Porto et al., 1977) and two species of *Galium*, as well as two other species within the Rubioideae as outgroups. For examination of species within *Relbunium*, exemplars were chosen to represent all sections and subsections recognized by Ehrendorfer (1955). All botanical material was deposited in the herbarium of the Rio de Janeiro Botanical Garden (RB), as shown in Table 1.

All material destined for optical microscopic analysis was fixed immediately after collection in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 (Gabriel, 1982), dehydrated in an ethanol series, embedded in Leica HistoResin (2-hydroxyethyl-metracrylate) (Nussloch, Germany), sectioned in a Shandon Hypercut microtome (Runcorn, Cheshire, U.K.) at 2–4 μm, and then stained with toluidine blue O 0.05% (O'Brien & McCully, 1981). Observations (in bright field) were made using an Olympus BX50 optical microscope (Center Valley, Pennsylvania, U.S.A.) equipped with a CoolSnap-pro digital camera (Houston, Texas, U.S.A.). Histochemical analyses were performed using Sudan III to

Table 1. Specimens examined. The vouchers are deposited at Rio de Janeiro Botanical Garden Herbarium (RB).

Taxa	Collection data
Galium latoramosum Clos	Brazil. Rio Grande do Sul: Ijuí, 24 Oct. 1984, *R. Bueno s.n.* (RB 444232); 30 Aug. 1904, *A. Bornmuller 132* (GH). Santa Catarina: Nova Teutônia, 3 Sep. 1944, *F. Plaumann 120* (RB).
G. uruguayense Bacigalupo	Brazil. Rio Grande do Sul: Caçapava do Sul, 3 May 2002, *K. De Toni s.n.* (RB 444168); Cristal do Sul, 20 Aug. 2001, *K. De Toni s.n.* (RB 444206); Guaíba, 11 Jan. 2003, *K. De Toni s.n.* (RB 444228).
Relbunium equisetoides (Cham. & Schltdl.) Ehrend.	Brazil. Rio Grande do Sul: Guaíba, 20 Feb. 2003, *N. I. Matzembacher s.n.* (RB 444212), *K. De Toni et al. s.n.* (RB 444210).
R. gracillimum Ehrend.	Brazil. Rio de Janeiro: Angra dos Reis, 22 Sep. 2004, *M. G. Bovini et al. 2460* (RB); Itatiaia, Mar. 2003, *K. De Toni et al. s.n.* (RB 4444211).
R. hirtum (Lam.) K. Schum.	Brazil. Rio Grande do Sul: Lavras do Sul, 4 May 2002, *K. De Toni et al. s.n.* (RB 444164).
R. humile (Cham. & Schltdl.) K. Schum.	Brazil. Rio Grande do Sul: Cambará do Sul, 9 Mar. 2002, *K. De Toni et al. s.n.* (RB 444192); 7 Apr. 2002, *K. De Toni et al. s.n.* (RB 444173); 26 Dec. 2002, *K. De Toni et al. s.n.* (RB 444216); Guaíba, 11 Jan. 2003, *K. De Toni et al. s.n.* (RB 444227); Jaquirana, 6 Apr. 2002, *K. De Toni et al. s.n.* (RB 444177); Maquiné, 26 Dec. 2002, *K. De Toni et al. s.n.* (RB 444217); Porto Alegre, 4 Oct. 2001, *K. De Toni et al. s.n.* (RB 444200); Tainhas, 29 Nov. 2001, *K. De Toni et al. s.n.* (RB 444197).
R. humilioides M. L. Porto & Ehrend.	Brazil. Rio Grande do Sul: Cambará do Sul, 9 Mar. 2002, *K. De Toni et al. s.n.* (RB 444188); 7 Apr. 2002, *K. De Toni et al. s.n.* (RB 444229).
R. hypocarpium (L.) Hemsl.	Brazil. Rio Grande do Sul: Barra do Ouro, 26 Dec. 2002, *K. De Toni et al. s.n.* (RB 444213); Caçapava do Sul, 3 June 2002, *K. De Toni et al. s.n.* (RB 444165, 444166); Cambará do Sul, 9 Mar. 2002, *K. De Toni et al. s.n.* (RB 444171); 29 Mar. 2002, *K. De Toni et al. s.n.* (RB 444178); 7 Apr. 2002, *K. De Toni et al. s.n.* (RB 444180); Porto Alegre, 6 Aug. 2001, *K. De Toni et al. s.n.* (RB 444207); Santo Antônio da Patrulha, 22 Aug. 2001, *K. De Toni et al. s.n.* (RB 444203); São José dos Ausentes, 10 Mar. 2002, *K. De Toni et al. s.n.* (RB 444184, 444185).
R. longipedunculatum Mariath & Ehrend.	Brazil. Rio Grande do Sul: Cambará do Sul, 9 Mar. 2002, *K. De Toni et al. s.n.* (RB 44189); 29 Mar. 2002, *K. De Toni et al. s.n.* (RB 444179); 7 Apr. 2002, *K. De Toni et al. s.n.* (RB 444170); 26 Dec. 2002, *K. De Toni et al. s.n.* (RB 444215); 5 Jan. 2004, *K. De Toni et al. s.n.* (RB 444160); Jaquirana, 6 Apr. 2002, *K. De Toni et al. s.n.* (RB 444175).
R. mazocarpum Greenm.	Brazil. Rio Grande do Sul: Cambará do Sul, 29 Mar. 2002, *K. De Toni et al. s.n.* (RB 444181).
R. megapotamicum (Spreng.) Ehrend.	Brazil. Rio Grande do Sul: Tainhas, 29 Mar. 2002, *K. De Toni et al. s.n.* (RB 444182); 29 Nov. 2002, *K. De Toni et al. s.n.* (RB 444196).
R. nigro-ramosum Ehrend.	Brazil. Rio Grande do Sul: Burro Novo, 6 Sep. 2004, *K. De Toni et al. s.n.* (RB 444230); Caçapava do Sul, 3 June 2002, *K. De Toni et al. s.n.* (RB 444167); Cambará do Sul, 9 Mar. 2002, *K. De Toni et al. s.n.* (RB 444187); 7 Apr. 2002, *K. De Toni et al. s.n.* (RB 444172); Jaquirana, 6 Apr. 2002, *K. De Toni et al. s.n.* (RB 444176); São Francisco de Paula, 29 Nov. 2001, *K. De Toni et al. s.n.* (RB 444198); 26 Dec. 2002, *K. De Toni et al. s.n.* (RB 444158); São José dos Ausentes, 10 Mar. 2002, *K. De Toni et al. s.n.* (RB 444186); Tainhas, 29 Nov. 2001, *K. De Toni et al. s.n.* (RB 444194).
R. ostenianum Standl.	Brazil. Rio Grande do Sul: Caçapava do Sul, 4 June 2002, *K. De Toni et al. s.n.* (RB 444224).
R. richardianum (Gillies ex Hook. & Arn.) Hicken	Brazil. Rio Grande do Sul: Cristal do Sul, 20 Aug. 2001, *K. De Toni et al. s.n.* (RB 444205); Lavras do Sul, 4 June 2002, *K. De Toni et al. s.n.* (RB 444163); Porto Alegre, 2 Aug. 2001, *K. De Toni et al. s.n.* (RB 444221); 16 Aug. 2001, *K. De Toni et al. s.n.* (RB 444222); Santo Antônio da Patrulha, 22 Aug. 2001, *K. De Toni et al. s.n.* (RB 444203); São Francisco de Paula, 9 Mar. 2002, *K. De Toni et al. s.n.* (RB 444193).
R. valantioides (Cham. & Schltdl.) K. Schum.	Brazil. Rio Grande do Sul: Santo Antônio da Patrulha, 22 Aug. 2001, *K. De Toni et al. s.n.* (RB 444195).
Borreria verticillata (L.) G. Mey.	Brazil. Rio Grande do Sul: Porto Alegre, *K. De Toni et al. 05* (RB).
Psychotria carthagenensis Jacq.	Brazil. Rio Grande do Sul: Porto Alegre, *K. De Toni et al. 06* (RB).

detect total lipids (O'Brien & McCully, 1981) and periodic acid Schiff (PAS) reaction to detect soluble polysaccharides (O'Brien & McCully, 1981).

Samples for SEM were fixed and then prepared according to the protocol described by Gersterberger and Leins (1978). Samples were dehydrated and prepared using a CPD 030 critical point dryer (Baltec, Schalksmühle, Germany), metal-coated using a Balzers SCD 050 sputtering system (Munich, Germany), and observed with a JEOL 5800 or DSEM 962 Zeiss scanning electron microscope (Oberkochen, Germany) with digital image acquisition.

Using morphological data (Appendix 1), a numerical matrix with 55 characters was constructed (Appendix 2). Parsimony analyses were performed using PAUP version 4.0b10 software (Swofford, 2002). The character states were treated as unordered, and the trees calculated using the neighbor-joining parsimony method. The confidence in the groups formed in these trees was verified using bootstrap and jackknife resampling tests with 1000 replicates for each analysis. The trees obtained were subsequently plotted and evaluated using the MacClade 4.0 software package (Maddison & Maddison, 2000).

RESULTS

FLOWERS

All the species had solitary flowers (cf. Appendix 2, character 2, state 0), except for *Galium latoramosum* Clos, *Relbunium equisetoides* (Cham. & Schltdl.) Ehrend., *R. richardianum* (Gillies ex Hook. & Arn.) Hicken, and the two outgroup taxa, which had compound dichasium, simple dichasium, and monochasium inflorescences.

All species of *Relbunium* examined had two, three, or four bracts. Species with four bracts had either two identical or unequal pairs (Fig. 1A–H); when unequal, there was one larger and one smaller pair (Fig. 1I, J). The most species have identical bracts (see Appendix 2, character 9, state 0); only two species present unequal pairs of bracts (Appendix 2, character 9, state 1); and three of them had only two bracts (Appendix 2, character 8, state 0; Fig. 1K–M), although a third bract was often observed in *R. ostenianum* Standl. (Appendix 2, character 8, state 1). Flowers generally had short peduncles (between 1 and 6 mm long) for 11 of the sampled species in *Galium* and *Relbunium* (cf. Appendix 2). Two species, *R. gracillimum* Ehrend. and *R. hypocarpium* (L.) Hemsl., demonstrated longer peduncles, to 15 mm. *Relbunium hirtum* (Lam.) K. Schum. and *R. ostenianum* had peduncles less than 1 mm long, and these flowers were considered sessile. Flowers were sessile in *R. equisetoides* and *R. richardianum*, while flowers of *G. latoramosum* had pedicels to 4 mm. Also, all of the species observed had bisporic flowers; *G. latoramosum*, however, had pistillate and staminate flowers on individual plants, which characterize monosporic flowers.

In terms of the floral whorls, seven species do not present unicellular trichomes on the abaxial face of the corolla (Appendix 2, character 17, state 0; Figs. 1A, E–G, I, J, N, 2A); four species present sparse trichomes on the abaxial face (Appendix 2, character 17, state 2; Figs. 1B, C, M, O, 2B, C); and also in four species unicellular trichomes were only observed along the corolla margins (Appendix 2, character 17, state 1; Figs. 1D, H, K, L, 2D). In addition to trichomes, idioblasts were observed on the abaxial faces of *Relbunium gracillimum* and *R. hypocarpium* (Fig. 2A; Appendix 2, character 22). These idioblasts were concentrated on the distal mid-third of the petals and were rectangular in shape, with dense cytoplasm and a distinct nucleus. Trichomes on the adaxial face were glandular and unicellular, situated near the margins and the central portion of the petals and, invariably, over the vascular bundles (Fig. 2E), or dispersed, as in *Galium uruguayense* Bacigalupo (Fig. 2B). These glandular trichomes were clavate (Fig. 2F, G), and a cuticle with randomly oriented low-ridge saliences (Fig. 2F) was present. During their early development, these trichomes have a large vacuole in their apical portions and a prominent nucleus. In later development, the large vacuole divides into many smaller ones (Fig. 2G). Histochemical tests indicated the presence of lipids and polysaccharides in the cytoplasm of these glandular trichomes.

The androecium is composed of four stamens with dorsifixed anthers, all of them tetrasporangiate (Fig. 2H), situated alternate to the petals and partially attached to them (Fig. 1A, C–O). At dehiscence, the anther walls reduce to only the epidermis and the endothecium (Fig. 2I). The endothecium had elongated cells and demonstrated unequal thickening of the lignified cell walls (Fig. 2I–K). These unequal thickenings appear as bands over the anticlinal walls (Fig. 2J) and as star-shaped (frontal view) over the internal periclinal walls (Fig. 2K). At anther dehiscence, pollen grains are bicellular or tricellular, with isopolar symmetry, forming pentacolpate or hexacolpate prolate spheroids or subprolate spheroids (Fig. 2L).

The gynoecium in *Relbunium* has a bicarpellate, bilocular inferior ovary, with one ovule per locule (Fig. 3A, B); however, tricarpellate ovaries were occasionally seen in *R. ostenianum* (Fig. 3C). The

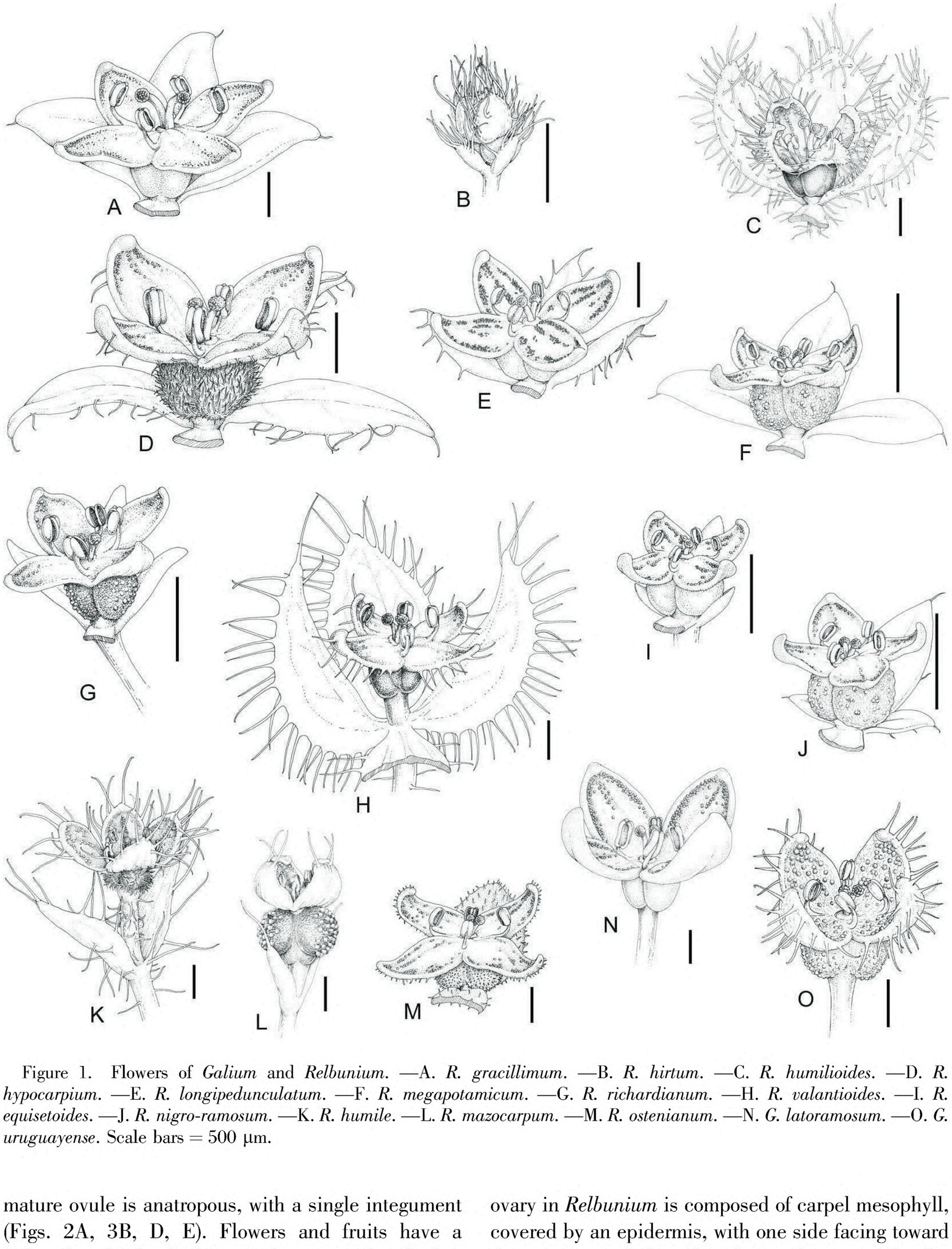

Figure 1. Flowers of *Galium* and *Relbunium*. —A. *R. gracillimum*. —B. *R. hirtum*. —C. *R. humilioides*. —D. *R. hypocarpium*. —E. *R. longipedunculatum*. —F. *R. megapotamicum*. —G. *R. richardianum*. —H. *R. valantioides*. —I. *R. equisetoides*. —J. *R. nigro-ramosum*. —K. *R. humile*. —L. *R. mazocarpum*. —M. *R. ostenianum*. —N. *G. latoramosum*. —O. *G. uruguayense*. Scale bars = 500 µm.

mature ovule is anatropous, with a single integument (Figs. 2A, 3B, D, E). Flowers and fruits have a reduced pedicel, which is similar to a peduncle, but lies between the bracts and the ovary. The reduced pedicel demonstrated varying lengths among the different species, being elongated (0.7–1.1 mm) in *R. humile* (Cham. & Schltdl.) K. Schum. (Fig. 3F) and *R. mazocarpum* Greenm. (Fig. 3G). This structure was not observed for the two species of *Galium* examined, as their flowers did not have bracts (Fig. 1N, O). The ovary in *Relbunium* is composed of carpel mesophyll, covered by an epidermis, with one side facing toward the exterior and the other facing the carpel locule. The external epidermis may have secretory idioblasts in addition to pavement cells (Figs. 2A, 3A), as well as unicellular trichomes (Fig. 3B, F, H). The external periclinal walls are covered with a cuticle that is diversely ornamented. In five species, rounded epidermal cells were observed in the external epidermis of the ovary (Fig. 3D; Appendix 2,

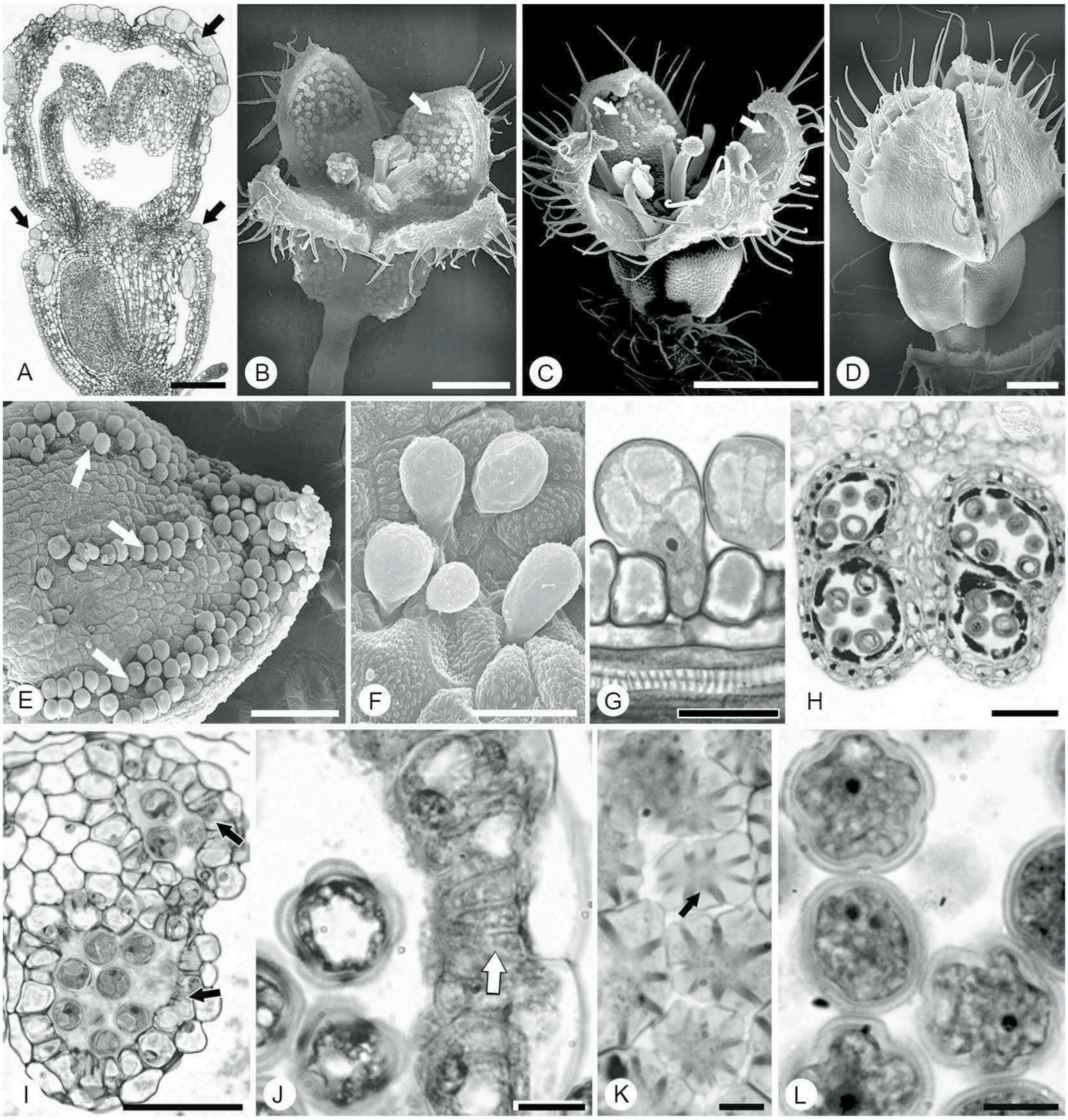

Figure 2. Flowers of *Galium* and *Relbunium*. —A. *R. gracillimum*. Flower bud, longitudinal section; corolla and ovary with idioblasts (arrows). —B. *G. uruguayense*. SEM of flower, glandular trichomes indicated (arrow). —C. *R. humilioides*. SEM of flower, evidencing glandular trichomes (arrows). —D. *R. valantioides*. SEM of flower bud. —E. *G. latoramosum*. SEM of corolla, adaxial view, with glandular trichomes (arrows). —F. *R. richardianum*. SEM of glandular trichomes of corolla (adaxial view). —G. *R. richardianum*. Glandular trichomes of corolla, transverse section (adaxial view). —H. *R. mazocarpum*. Anther tetrasporangiate, transverse section. —I. *R. hirtum*. Anther, transverse section, with endothecium cells (arrows). —J. *R. nigro-ramosum*. Anther details, transverse section, evidencing the endothecium (arrows). —K. *R. hirtum*. Frontal view of endothecium cells, with unequal wall thickness (arrow). —L. *R. hirtum*. Pollen grain detail. Scale bars: F, J–L = 50 μm; G, H, I = 100 μm; B, E = 200 μm; A, C, D = 500 μm.

character 26). In *R. hirtum*, these epidermal cells are secretory, as the cuticle became deformed by the accumulation of secretions between it and the external periclinal wall (Fig. 3E).

The secretory idioblasts of *Galium uruguayense* (Fig. 3I; cf. also Appendix 2, character 30), *Relbunium richardianum*, *R. gracillimum* (Fig. 2A), *R. nigro-ramosum* Ehrend. (Fig. 3A), *R. megapotamicum* (Spreng.) Ehrend. (Fig. 3J), and *R. mazocarpum* were scattered over the external surface of the epidermis, except for the region near the ovary septum (Fig. 3G). In *R. gracillimum* (Fig. 2A), idioblasts were observed only on the superior portion of the ovary, near the corolla. Unicellular trichomes were observed in *R. ostenianum* (Fig. 3B), *R. humile* (Fig. 3F), and *R. hypocarpium* (Fig. 3H).

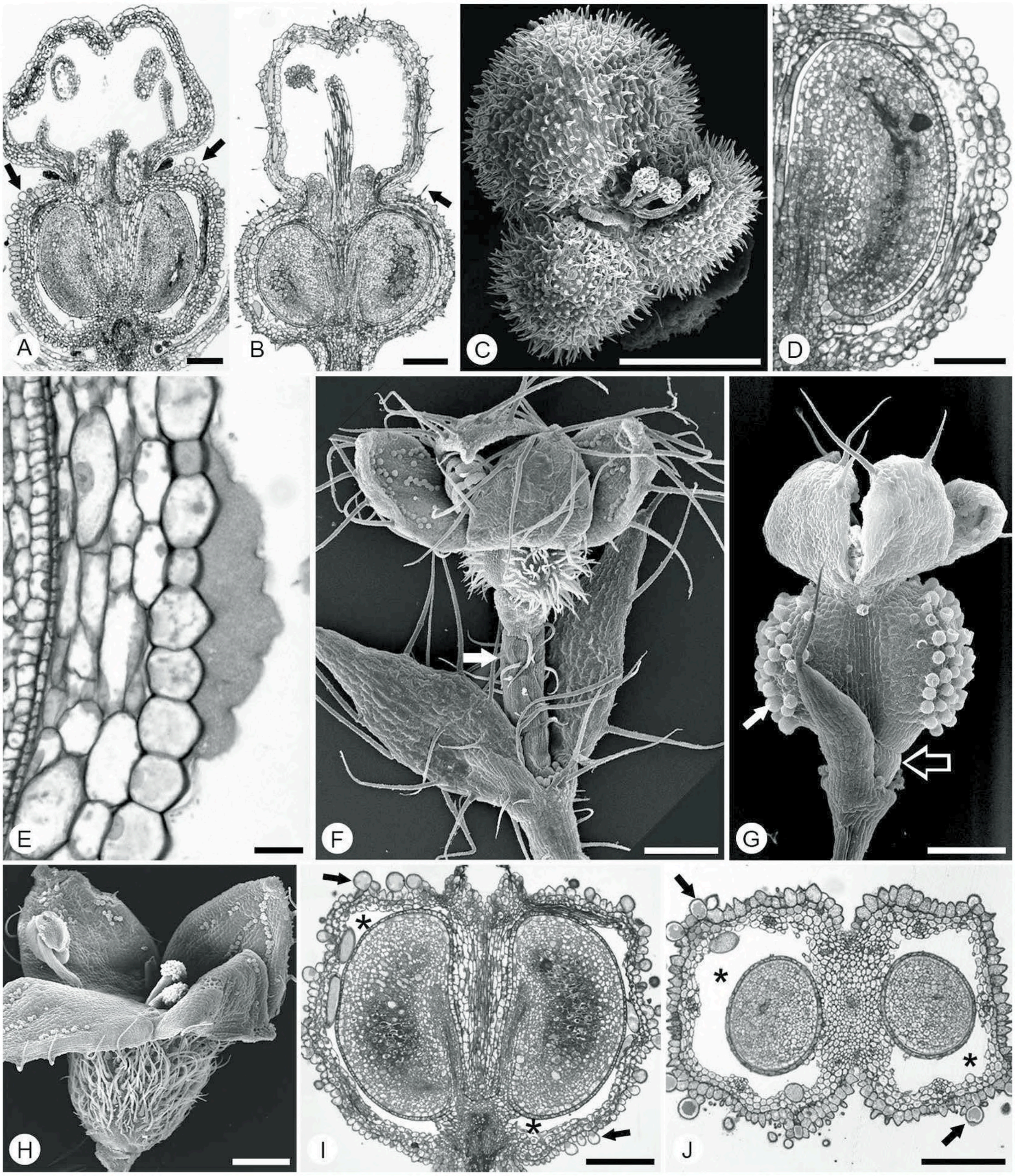

Figure 3. Flowers of *Galium* and *Relbunium*. —A. *R. nigro-ramosum*. Flower bud, longitudinal section, with idioblasts (arrows). —B. *R. ostenianum*. Flower bud, longitudinal section, with unicellular trichomes (arrow). —C. *R. ostenianum*. SEM of ovary after fertilization. —D. *R. humilioides*. Ovule, longitudinal section. —E. *R. hirtum*. Ovary wall with secretion accumulation (transverse section). —F. *R. humile*. SEM of flower with reduced pedicel (arrow). —G. *R. mazocarpum*. SEM of flower with reduced pedicel (black arrow) and idioblasts (white arrow). —H. *R. hypocarpium*. SEM of flower. —I. *G. uruguayense*. Ovary after fertilization, longitudinal section, with idioblasts (arrow) and pericarp lacunae (asterisks). —J. *R. megapotamicum*. Ovary, after fertilization, transverse section, with idioblasts (arrows) and pericarp lacunae (asterisks). Scale bars: D, E = 100 μm; J = 200 μm; A–C, F–I = 500 μm.

Periclinal divisions were observed in the carpel mesophyll of *Galium latoramosum*, in addition to the usual anticlinal divisions. Some cells on the external face of the epidermis divided in the periclinal plane, but did not constitute a bistratified epidermis. In both *Galium* and *Relbunium* species, the epidermis of the internal ovary face was invariably unistratified, having cells similar to those on the external epidermis, although smaller in size. This internal layer remained adjacent to the ovule. Before

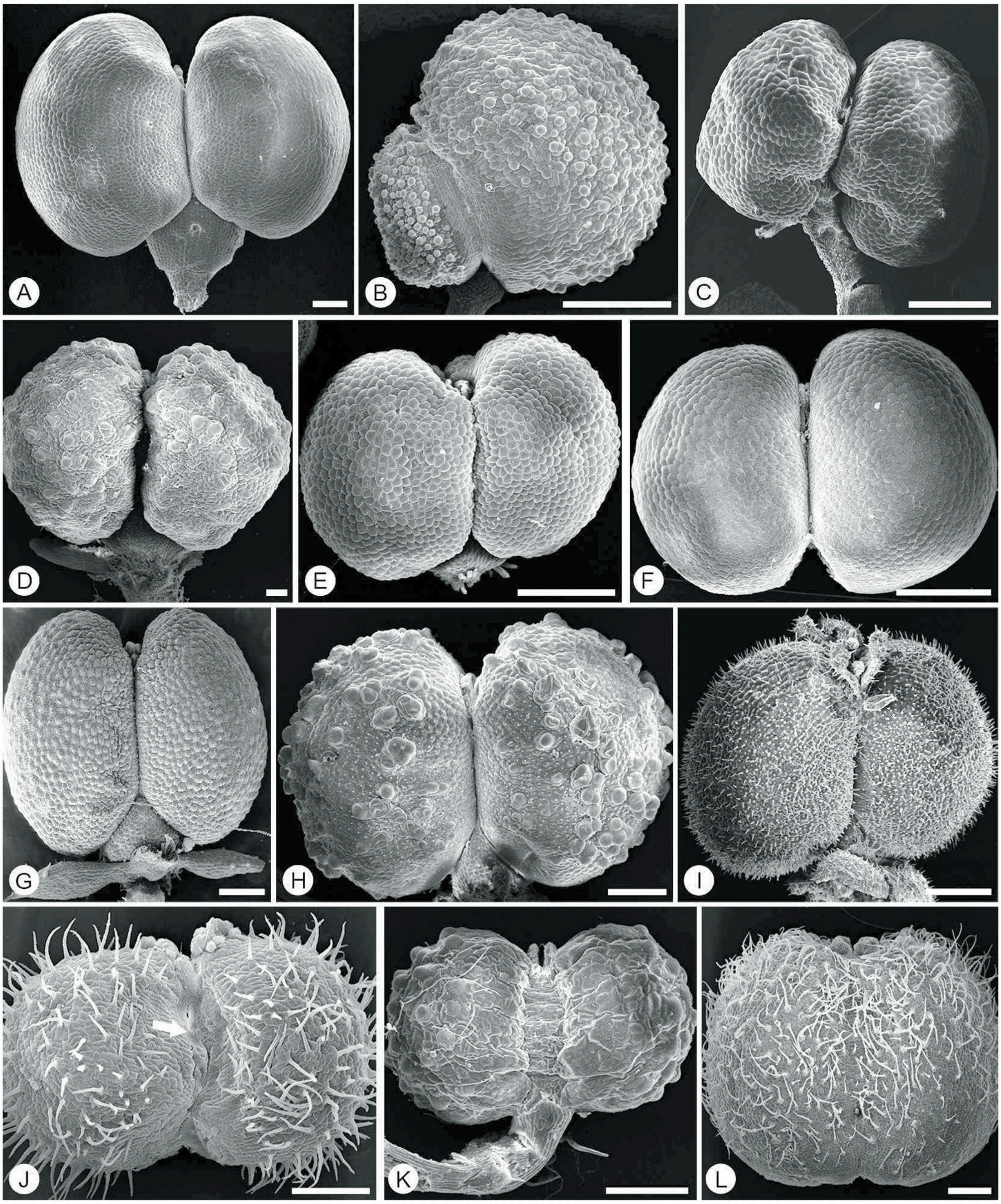

Figure 4. SEM fruits of *Galium* and *Relbunium*. —A. *R. valantioides*. —B. *G. uruguayense*. —C. *R. equisetoides*. —D. *R. nigro-ramosum*. —E. *R. humilioides*. —F. *R. hirtum*. —G. *R. longipedunculatum*. —H. *R. megapotamicum*. —I. *R. ostenianum*. —J. *R. humile*. Mericarp abscission region (arrows). —K. *R. mazocarpum*. —L. *R. hypocarpium*. Scale bars = 500 μm.

fertilization, large lacunae were formed by cellular lysis in the carpel mesophyll (Fig. 3I, J).

FRUITS

Fertilization may occur simultaneously in the two ovules or in only one; in both cases, fruits are formed, having either two (Fig. 4A) or only one mericarp (Fig. 4B), respectively. Two types of fruits were observed in *Relbunium*, schizocarps (Fig. 4A–K) and berries (Fig. 4L), with the latter only seen in *R. gracillimum* and *R. hypocarpium*. The early development of the two fruits was similar, differing relative to the pericarp succulence.

The tuberculate aspect of the fruit (Fig. 4B, D, H, K) in *Galium uruguayense* and three *Relbunium* species results from the presence of secretory idioblasts (Fig. 5A) covered by short striae of varying thicknesses (Fig. 5B). In *R. nigro-ramosum* and *R. megapotamicum*, the idioblasts are prominent in relation to the epidermis, and they are supported by two to three epidermal cells as well as mesocarp cells (Fig. 5C). The idioblasts occur isolated or in groups and range in size from five (*R. nigro-ramosum*, Fig. 4D) to 10 cells (*R. megapotamicum*, Fig. 4H). The cytoplasm of these cells appears very dense (Fig. 5A, C), indicating secretory activity. In *R. richardianum*, the cuticle becomes displaced by the accumulation of secretions between it and the external periclinal exocarp wall (Fig. 5G). Additional modifications of the other exocarp cells and cuticle were observed as the fruit continued to develop and the saliences of cuticle ridges and wrinkles (Fig. 5D) became distended and sparser (Fig. 5E). In *G. latoramosum*, the exocarp cells do not have ornamented cuticles (Fig. 5F).

In addition to idioblasts, unicellular trichomes were also observed on the exocarp of *Relbunium ostenianum* (Fig. 4I), *R. humile* (Fig. 4J), and *R. hypocarpium* (Fig. 4L). Other species, however, demonstrated fruits without idioblasts or trichomes, including *R. equisetoides* (Fig. 4C), *R. hirtum* (Fig. 4F), *R. humilioides* M. L. Porto & Ehrend. (Fig. 4E), *R. longipedunculatum* Mariath & Ehrend. (Fig. 4G), and *R. valantioides* (Cham. & Schltdl.) K. Schum. (Fig. 4A).

In early developmental stages, the mesocarp resembles the carpel mesophyll, except in *Galium latoramosum* and *Relbunium longipedunculatum*, which demonstrated an increase in cell layers, from five to eight, due to periclinal divisions (Fig. 5H). Until this stage is reached, the development of the two fruit types in *Relbunium* (schizocarps and berries) is similar. As seeds mature, however, structural differences can be observed in the pericarp, and these characteristics are therefore important in distinguishing among species of *Relbunium*.

In later developmental stages, the mesocarp of the schizocarpic fruit in *Relbunium* (Fig. 4A–K) reveals elongated cells with thin, and frequently obliterated, primary cell walls (Fig. 5K). Idioblasts with raphide crystals can be observed adjacent to the endocarp. In *R. equisetoides*, the mesocarp retains characteristics similar to the palisade parenchyma (Fig. 5J), as previously described for the ovary.

Similar to the exocarp, the endocarp cells slowly elongate during fruit maturation until the seed completes its development. The endocarp appeared greatly distended in *Galium uruguayense*, *Relbunium equisetoides*, *R. humilioides*, *R. valantioides*, *R. longipedunculatum*, and *R. megapotamicum*, although it remained intact and visible (Fig. 5I). Although the endocarp remained intact in *R. nigro-ramosum* and *R. ostenianum*, it was later almost imperceptible between the seed coat and the mesocarp (Fig. 5K). A discontinuity of the endocarp was observed in *G. latoramosum*, *R. richardianum*, *R. hirtum*, *R. humile*, and *R. mazocarpum*, in which the endocarp ruptured during fruit development and became obliterated and imperceptible (Fig. 5L).

During seed development, cellular digestion of the endosperm by the integument (Fig. 5M) and the endosperm by the embryo was observed, forming an endosperm digestion cavity (Fig. 5N). The integument was eventually almost entirely consumed, leaving only a single cell layer that formerly composed its external epidermis, or exotesta (Fig. 5I–K, O). The cytoplasm of the exotesta cells contained phenolic compounds in *Relbunium hirtum* (Fig. 5L), *R. longipedunculatum*, and *R. ostenianum*.

The endosperm appeared to be composed of homogeneous cells (Fig. 6A) with thickened cell walls composed principally of cellulose and pectins; significant quantities of lipids were observed in their cytoplasm. Additionally, a gradient of the cell wall thickness was observed in the endosperm, with the peripheral cells being much thicker than those closest to the embryo (Figs. 5L, 6B).

After embryo maturation, the schizocarpic fruit dehisces by the separation of the mericarps along an abscission line that divides them (Figs. 4J, 5N, 6A). This abscission region formed during the ovary development, with the contact between the two carpels giving rise to a zone of fragility. The cells in this region are parenchymatic, elongated, and have thin cell walls (Fig. 6C). Like schizocarpic fruits, berries also have distended exocarp and endocarp cells during development and maturation.

Idioblasts present on the external ovary epidermis (Fig. 2A) during the immature stages of the berry fruits of *Relbunium gracillimum* were not retained in the mature fruit (Fig. 6D), as fruit growth stretches the exocarp cells. However, trichomes present on the ovary and immature fruits of *R. hypocarpium* could still be observed on the mature fruits of this species (Fig. 4L).

The mesocarp appears succulent, having large, isodiametric parenchymatic cells with very thin cell walls and large vacuoles (Fig. 6E). Intercellular spaces were observed in *Relbunium hypocarpium*, but were absent in *R. gracillimum*. When the embryo reaches the torpedo stage, the mesocarp is composed

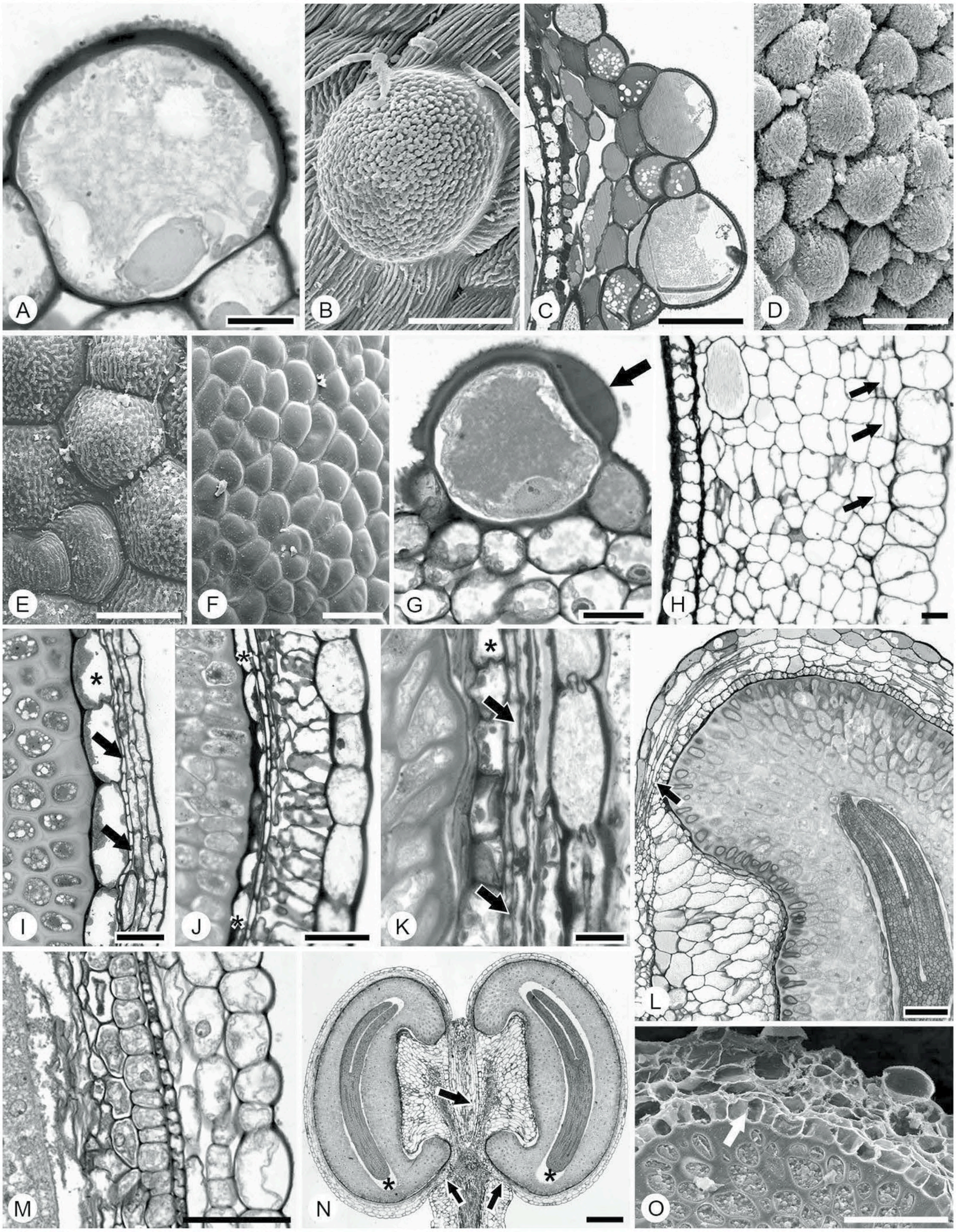

Figure 5. Fruits of *Galium* and *Relbunium*. —A. *G. uruguayense*. Idioblast detail, longitudinal section. —B. *G. uruguayense*. SEM of idioblast, frontal view. —C. *R. megapotamicum*. Pericarp with prominent idioblasts, longitudinal section. —D. *R. longipedunculatum*. SEM of epicuticular striae on exocarp, frontal view. —E. *R. longipedunculatum*. SEM of exocarp with epicuticular striae. —F. *G. latoramosum*. SEM of exocarp, frontal view. —G. *R. richardianum*. Secretion accumulation in idioblast (arrow), longitudinal section. —H. *G. latoramosum*. Pericarp with periclinal divisions (arrows), transverse section. —I. *G. uruguayense*. Pericarp evidencing endocarp (arrows) and seed coat (asterisk), transverse section. —J. *R. equisetoides*. General view of pericarp and seed coat (asterisks), transverse section. —K. *R. ostenianum*. Pericarp with endocarp (arrows) and seed coat (asterisk), transverse section. —L. *R. hirtum*. Mericarp section, with endocarp (arrows), longitudinal section. —M. *R. valantioides*. Early pericarp detail, transverse section, evidencing endosperm on integument cellular digestion. —N. *R. valantioides*. General fruit aspect, longitudinal section, with digestive cavity (asterisks) and abscission region (arrows). —O. *G. uruguayense*. Pericarp detail with seed coat (arrow). Scale bars: A, B, E, G = 50 µm; C, D, F, H–M, O = 100 µm; N = 500 µm.

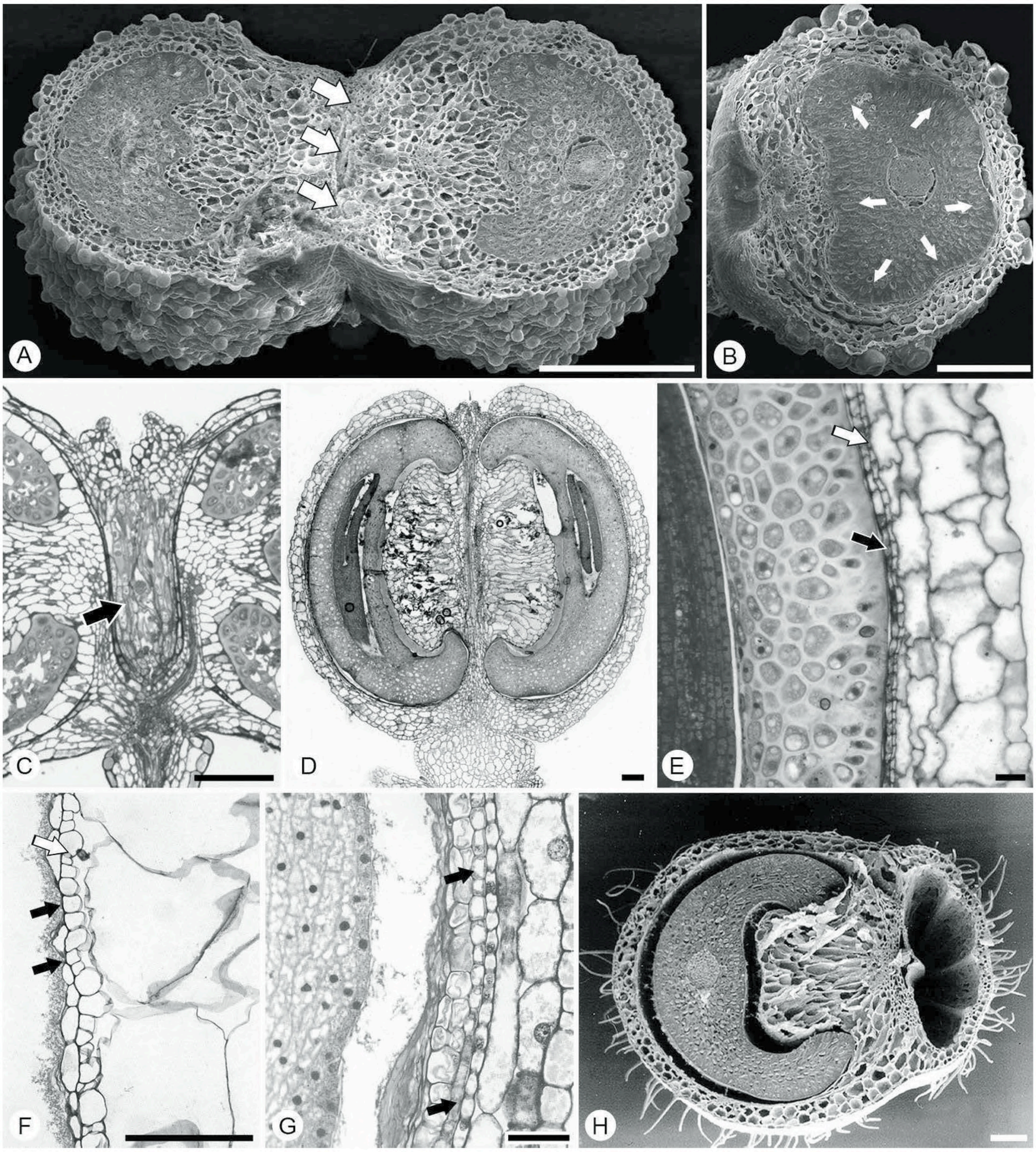

Figure 6. Fruits of *Galium* and *Relbunium*. —A. *G. uruguayense*. SEM of fruit, transverse section, with abscission region (arrows). —B. *R. megapotamicum*. SEM of mericarp, transverse section, endosperm cells with distinct wall thickness (arrows indicate edge of thicker cell walls). —C. *R. mazocarpum*. Fruit, longitudinal section, with mericarp abscission region detail (arrow). —D. *R. gracillimum*. Fruit, longitudinal section. —E. *R. gracillimum*. Pericarp, longitudinal section, with endocarp (white arrow) and seed coat (black arrow). —F. *R. hypocarpium*. Seed coat detail, transverse section, with fragmented portions (black arrows) and endocarp (white arrow). —G. *R. hypocarpium*. Pericarp detail, transverse section, with endosperm on integument cellular digestion. Cell layer that constitutes the exotesta (arrows). —H. *R. hypocarpium*. SEM of fruit, transverse section, with only one seed. Scale bars: F = 50 μm; C, E, G, H = 100 μm; A = 200 μm; B, D = 500 μm.

of three to five cell layers (Fig. 6D). Idioblasts with raphide crystals could be observed adjacent to the endocarp. The endocarp of the mature fruit remains intact, even after experiencing distension (Fig. 6E, F).

The exotesta was observed to form during endosperm digestion of the integument (Fig. 6G). During this phase, the ovule cell layers are digested, leaving only a single layer that covers the dorsal and ventral faces of the seed of *Relbunium gracillimum* (Fig. 6E). As in *R. gracillimum*, the exotesta of *R. hypocarpium* is composed of only a single cell layer, and it ruptures as the fruit grows, leaving fragments attached to the endocarp (Fig. 6F). *Relbunium hypocarpium* is the only *Relbunium* species in which the seed is not uniformly enveloped by the exotesta.

The seeds of both schizocarp and berry fruits are elliptical to spherical, but schizocarp seeds have a slight sinuosity (Fig. 6B) not seen in berry seeds (Fig. 6H). A depression resulting from residual funicular tissue, which was not digested during endosperm formation, can be seen in the ventral region of the seeds (Fig. 6A, H). The torpedo phase embryo is curved and completely enveloped by the endosperm (Fig. 5N).

PHYLOGENETIC ANALYSES

Morphological data (Appendix 1) were used for parsimony analysis. The matrix comprised a total of 55 characters (Appendix 2). Of these 55 characters, six were considered variable, but without informative value for phylogenetic analysis, while 46 were considered parsimony informative. Parsimony analysis produced a phylogenetic tree 262 steps long, with a consistency index of 0.47 and a retention index of 0.51 (Fig. 7). This cladogram distinguished between the genera *Galium* and *Relbunium* based on the presence/absence of the fertile bracts, and the branch that included the two species of *Galium* was sister to all sampled *Relbunium*. Both clades share glandular trichomes on the adaxial corolla faces, schizocarpic fruits, and pericarp lacunae. The *Relbunium* clade is defined by the presence of a reduced pedicel and consists of two subclades (A and B), distinguished by having either four or two bracts, respectively.

Clade A in *Relbunium* is further partitioned into two species groupings—clade A1 and A2 (Fig. 7). The A1 species have imperceptibly reduced pedicels, while species of clade A2 have pedicels less than 0.7 mm long. *Relbunium equisetoides* lies basal in clade A1. This species differs from others in that the abaxial corolla face is glabrous and the fruit exocarp is ornamented with small saliences of cuticle ridges. The remaining species group of six shares an exocarp with a striate cuticle. Affined species consist of *R. richardianum*, *R. nigro-ramosum*, and *R. megapotamicum*. Two of these species (*R. nigro-ramosum* and *R. megapotamicum*) have fruits with parallel cuticular striae oriented toward the center of the epidermal cells forming a cuticular salience, as well as secretory idioblasts on the exocarp that are either isolated or united into clusters. *Relbunium richardianum* lies basal to these two species. *Relbunium nigro-ramosum* has bracts that are unequal in size, while the bracts of *R. megapotamicum* are uniform. A second affined group in clade A1 is united by the presence of tannin in the seed coat (*R. hirtum*, *R. ostenianum*, and *R. longipedunculatum*). The latter species differs in having pedunculate flowers with peduncles to 10–15 mm, and the fruit endocarp is continuous and remains intact during the entire phase of seed development. Sessile flowers and discontinuous endocarps distinguish both *R. hirtum* and *R. ostenianum*; however, they overtly differ in that *R. ostenianum* has two (or three) fertile bracts, while *R. hirtum* has four.

The four species that comprise clade A2 in *Relbunium* (Fig. 7) share an evident reduced pedicel and divide into two subgroups. The first consists of the species pair *R. gracillimum* and *R. hypocarpium*, with fleshy berries, while *R. humilioides* and *R. valantioides* have schizocarpic fruits. *Relbunium hypocarpium* differs from *R. gracillimum* in having unicellular trichomes on the external ovary face. *Relbunium humilioides* diverges from *R. valantioides* in having dispersed trichomes on the abaxial corolla face, ample intercellular spaces in the mesocarp, and an exocarp with short striae on the cuticle with no apparent orientation pattern. In contrast, in *R. valantioides*, the trichomes restrict to the margins of the abaxial corolla face, the intercellular spaces in the mesocarp are reduced and infrequent, and the exocarp cuticle is organized into long, parallel striae.

Clade B in *Relbunium* (Fig. 7) is composed of only two species, *R. humile* and *R. mazocarpum*. The pair lie sister to the other 11 exemplar species in *Relbunium* and basically differ by the presence of only two bracts and a prominent reduced pedicel. Of the pair, *R. mazocarpum* has fruits with secretory idioblasts on the exocarp, while in *R. humile* the exocarp is only pilose.

DISCUSSION

Despite studies focused on the genera *Galium* and *Relbunium*, their generic boundaries and respective species remain under discussion due to their significant morphological similarities. Additionally, many characteristics often used to distinguish the taxa of Rubiaceae are morphologically plastic and variable in response to their environments (Puff, 1975). Despite this, reproductive characteristics may remain consistently and systematically useful (Herr, 1984).

Galium has been distinguished by the following characteristics: a herbaceous and subshrubby habit; quadrangular stems; chartaceous to membranaceous leaves, with or without revolute margins (Dempster & Ehrendorfer, 1965); nodes with two opposite leaves, and two or more foliar stipules (Dempster, 1982); and

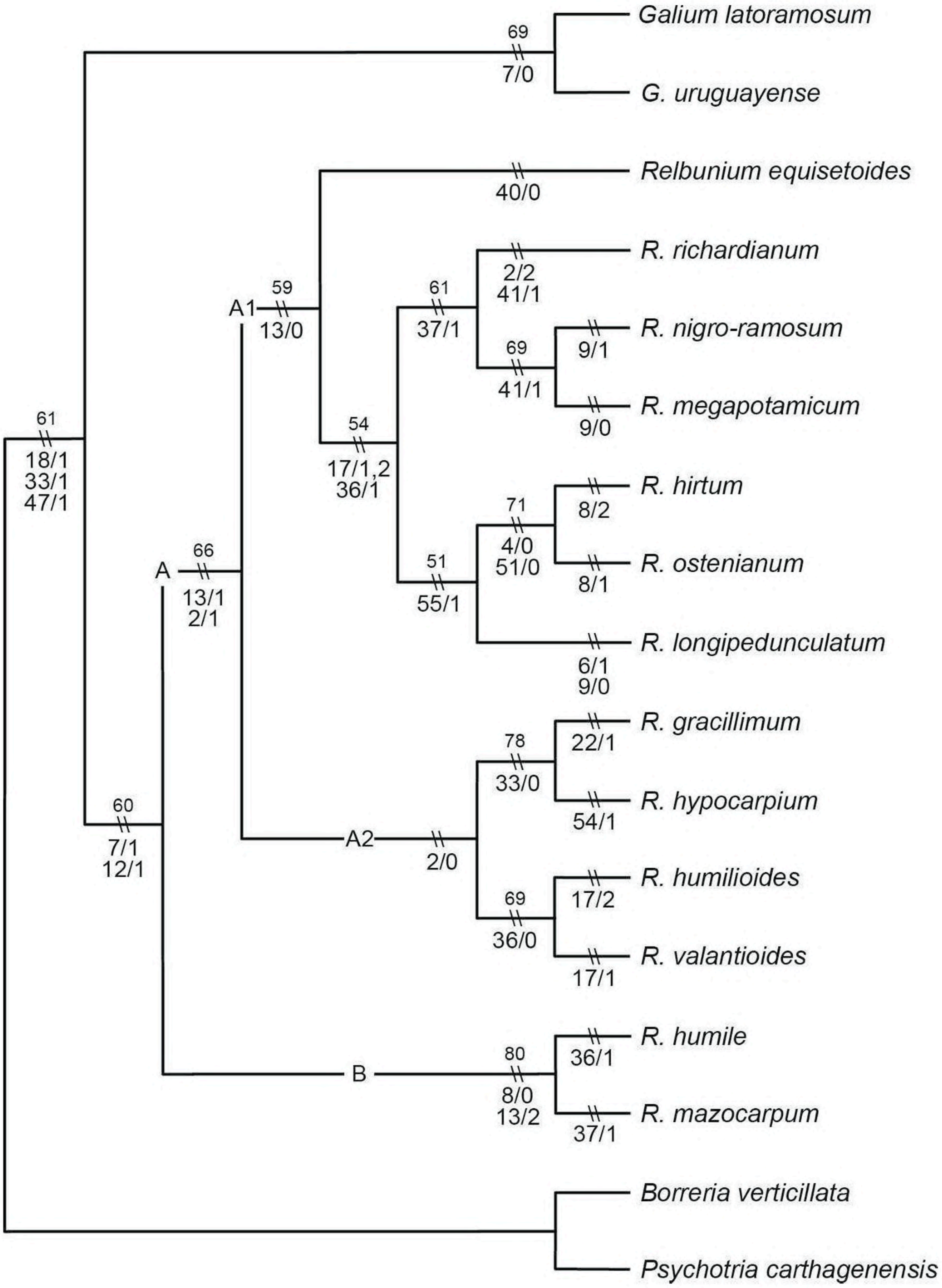

Figure 7. Parsimonious tree of *Galium* and *Relbunium* species derived from morphological data. Numbers above branches denote bootstrap percentages from 1000 replicates.

trichomes that are diminutive, retrorse, and more or less aculeate on the stems and leaves, but uncinate in the fruits (Dempster, 1981). The genus *Relbunium* is vegetatively similar to *Galium*. As such, reproductive morphologies can distinguish these two genera (Schumann, 1891; Ehrendorfer, 1955; Dempster, 1978).

In Rubiaceae, the basic inflorescence is the thyrse (Weberling, 1977), which is considered a plesiomorphic character within the family. Only the presence of solitary flowers, such as in *Relbunium*, might constitute a synapomorphy. However, in *Relbunium*, the appearance of inflorescences in two species may characterize a reversion, as observed in *R. equisetoides* and *R. richardianum*.

According to Robbrecht (1988), bracts are found in a majority of the inflorescences in Rubiaceae. Differing from many North American species, European species of *Galium* lack bracted inflorescences (Ehrendorfer, 1955). Species of *Galium* may sometimes have one or two bracts, such as *G. correllii* Dempster (Dempster, 1968), *G. wigginsii* Dempster

(Dempster, 1970), *G. ecuadoricum* Dempster, *G. ovalleanum* Phil., *G. trichocarpum* DC., *G. philippianum* Dempster (Dempster, 1980), *G. peruvianum* Dempster & Ehrend., *G. ferrugineum* K. Krause (Dempster, 1981), *G. araucanum* Phil., and *G. suffruticosum* Hook. & Arn. (Dempster, 1982). These species occur in North and Central America, with some to the western edge of the Andes Mountains to the south (Dempster, 1970, 1980, 1981, 1982).

This study documents the absence of bracts in *Galium latoramosum* and *G. uruguayense*. However, considering the presence of bracts in *Relbunium*, it should be noted that no variations of this characteristic were observed. Ehrendorfer (1955) and Detoni (1976) have suggested that the species of the genus *Relbunium* originated from New World species of *Galium*. Based on this hypothesis, we may infer that (1) the ancestors of *Relbunium* did not have bracts, and (2) because there is a north to south gradient in the number of bracts in the species of *Galium* through those American mountain ranges, the species that spread southward along the Andes gradually acquired them. Some of the species in northern California (U.S.A.) have one (or no) bracts (Dempster, 1968), while species from the southern region of this same state (Dempster, 1970, 1975) and in northern Mexico (Dempster, 1978) may have one to two bracts, and the number of bracts continues to increase along the Andes mountain chain, varying from two to four along its north to south distributional axis (Dempster, 1980, 1981, 1982).

As the presence of involucrate inflorescences is common in Rubiaceae (Robbrecht, 1988), it may be assumed that the absence of bracts in species of *Galium* represents a synapomorphy for that group, and consequently the presence of bracts in *Relbunium* represents a reversion with bracts separated from the flower by a reduced pedicel. These reduced pedicels were observed in the 13 species of *Relbunium* examined, although their lengths varied. From the cladogram (Fig. 7), it can be seen that the presence of a reduced pedicel is synapomorphic for the clade formed by *R. humile* and *R. mazocarpum*. Species with a prominent reduced pedicel were considered a basal group in *Relbunium*, indicating that the tendency toward elongating these structures is not a derived condition, but rather a plesiomorphic characteristic of the genus.

According to Ehrendorfer (1955), species of *Galium* do not have bracts, the flowers are overtly pedunculate, exceeding 15 mm, and the fruits have, in many cases, specialized trichomes. On the other hand, in *Relbunium*, the flowers have two to four bracts, are sessile or have short peduncles, and specialized trichomes are not observed on the fruits. Contrasted with observations of Ehrendorfer (1955), the present work noted the presence of long peduncles, at least 1.8 mm, on *Relbunium* flowers. However, *R. hirtum* and *R. ostenianum* demonstrated sessile flowers, thus confirming the importance of this characteristic to the phylogeny of the genus in the cladogram (Fig. 7).

Despite the fact that characters related to carpel development did not reveal differences between Rubiaceae taxa, fruit characteristics did indicate differences between the species of *Galium* and *Relbunium* analyzed. Different authors have described two types of pericarps: fleshy and dry. According to Eames and MacDaniels (1953), fruits with a fleshy pericarp are succulent and have parenchyma cells with thin walls that accumulate water in their vacuoles. Ergastic substances, such as starches, oils, and proteins, are frequently present, as well as calcium oxalate crystals and tannins (Roth, 1977). Based on these characteristics, among the species examined, only *G. latoramosum*, *R. gracillimum*, and *R. hypocarpium* have a fleshy pericarp. Fruits with a dry pericarp do not have a fleshy consistency (Font Quer, 1985), but do have sclerenchyma cells (Eames & MacDaniels, 1953) and dead cells when mature (Roth, 1977). However, the mesocarp of *G. uruguayense* and the *Relbunium* species cannot be considered dry according to this definition because they do not have cells with lignified cell walls and/or dead cells. These fruits have elongated cells with thin walls that remain alive and do not show secondary thickening, without, however, being succulent.

It is difficult to define the pericarps of the analyzed species as fleshy or dry, because they share characteristics with both and are considered intermediate. The mesocarp of *Pagameopsis* Steyerm. was considered to be more or less fleshy (Piesschaert et al., 2001). The pericarps of species of *Pentanisia* Harv. were described as rather succulent, although they have lignified portions (Puff & Robbrecht, 1989). In many Gardeniinae, the pericarp has been described as more or less dry (Robbrecht, 1988). According to Spjut (1994), the pericarp of fruits may sometimes be slightly fleshy or dry. Dempster (1990) indicated that the *Galium* fruits are relatively dry and that truly dry fruits are probably not found in this genus. Apparently, the fruits of *G. uruguayense* and the species of *Relbunium* are intermediate types with slightly dry pericarps (except for *G. latoramosum*, *R. gracillimum*, and *R. hypocarpium*, which have fleshy pericarps).

Rubiaceae demonstrate high morphological diversity in terms of its fruits, and phylogenetic studies have suggested that the ancestral fruit of the family was a many-seeded, dry capsule. It was then proposed by Bremer and Eriksson (1992) that fleshy fruits have arisen at least 12 times within the family, with four instances in Rubioideae and only one in Rubieae. Within this tribe, the tendency toward fleshiness is observed in *Didymaea* Hook. f., *Rubia*, and in some species of *Galium*. In *Galium*, and in related genera, fleshy fruits may have arisen from dry fruits after a reduction in seed number (Bremer & Eriksson, 1992). According to Bremer and Eriksson (1992), in *Relbunium gracillimum* and *R. hypocarpium* the fleshy fruits arose from dry fruits, being exclusive to and as a synapomorphy for the genus.

In comparing the pericarps of the *Relbunium* species analyzed here with those of *Rubia peregrina* L., *R. tinctorum* L., *Galium aparine* L., and *G. mollugo* L. described by Goursat and Guignard (1975), a transition from fleshy to dry pericarps can be inferred. *Rubia* is considered a sister group of the tribe (Manen et al., 1994), and the mesocarps of its species have many cell layers and a fleshy aspect, while in *Relbunium* there are fewer mesocarp layers and the fruits do not appear succulent. The number of mesocarp layers is even more reduced in *G. aparine* and *G. mollugo* (not more than three), and their fruits are schizocarps, although with a fleshy pericarp.

Fleshy and dry fruits, capsules, schizocarps, drupes, and berries are encountered within the Rubiaceae (Barroso et al., 1999). Roth (1977) classified the fruits of *Galium* as schizocarps, in accordance with Barroso et al. (1999), for the species of *Relbunium*. Schizocarpic fruits were first described by Schleiden (1837) for Apiaceae. Spjut (1994: 28–29) defined the fruits of *Galium* as an achenarium, a term that is synonymous with schizocarp, i.e., fruits that are derived from a schizocarp gynoecium, with indehiscent carpels that separate and represent distinct dispersal units (the mericarp). Many authors define schizocarp fruits as dehiscent, but this affirmation is conceptually equivocal. Because the pericarp does not rupture, with only a separation of the carpels being observed, they are, in fact, indehiscent (Spjut, 1994). Schizocarpic fruits in Rubiaceae are otherwise found in species of *Neopentanisia* Verdc. (Puff & Robbrecht, 1989) and in the genus *Scyphiphora* C. F. Gaertn. (Puff & Rohrhofer, 1993).

In addition to schizocarpic fruits, berries were observed in *Relbunium*, in *R. gracillimum* and *R. hypocarpium*. Berries, according to Barroso et al. (1999), are indehiscent fruits with fleshy and thin pericarps and undifferentiated endocarps. In the Rubiaceae, this type of fruit has been described for *Salzmannia* DC., *Coussarea* Aubl., *Faramea* Aubl., *Coccocypselum* P. Browne, *Schradera* Vahl, *Hippotis* Ruiz & Pav., *Hoffmannia* Sw., *Sabicea* Aubl., *Bertiera* Aubl., *Hamelia* Jacq., *Stachyarrhena* Hook. f., *Posoqueria* Aubl., *Gardenia* J. Ellis, *Randia* L., *Tocoyena* Aubl., *Genipa* L., *Amaioua* Aubl., *Melanopsidium* Colla, *Alibertia* A. Rich., *Borojoa* Cuatrec., *Kutchubaea* Fisch. ex DC., and *Duroia* L. f. (Barroso et al., 1999).

Variation was observed in both the seed morphology and the seed number per locule within Rubiaceae. The seed coat of Rubiaceae seeds is composed of an exotesta. This exotesta is composed of more than one cell layer in species of *Paragenipa* Baill. and *Didymosalpinx* Keay, although other genera have a reduced or absent exotesta (Robbrecht, 1988). When reduced, the exotesta appears as a membranaceous layer of cells with thin cell walls, located between the pericarp and the endosperm, as in *Callipeltis cucullaris* (L.) DC., *Sherardia arvensis* L. (Lloyd, 1902), *Oldenlandia alata* Roxb., *Dentella repens* (L.) J. R. Forst. & G. Forst. (Raghavan & Rangaswamy, 1941), *Borreria hispida* Spruce ex K. Schum. (Farooq, 1952), *O. corymbosa* L., *O. nudicaulis* Roth (Farooq, 1953), *Randia malabarica* Lam. (Periasamy, 1962), *Tarenna asiatica* Kuntze ex K. Schum. (Periasamy, 1964), *Anotis lancifolia* Hook. f. (Ahmad, 1978), *Dentella repens*, *D. serpyllifolia* Wall. ex Craib (Maheshwari & Raju, 1980), *Macrosphyra* Hook. f., *Argocoffeopsis* Lebrun, *Calycosiphonia* Robbr., *Craterispermum* Benth., and the Aulacocalyceae tribe (Robbrecht, 1986). Mariath (1990) described the seeds of *Relbunium hypocarpium* as lacking a seed coat, although De Toni and Mariath (1999) described a thin and sometimes discontinuous layer of cells corresponding to the exotesta for the same taxa. In analyzing the species of *Galium* and *Relbunium* in this paper, the presence of at least one cell layer covering the endosperm was confirmed, thus characterizing an exotesta, in agreement with De Toni and Mariath (1999).

Manen et al. (1994) characterized the tribe Rubieae as monophyletic and recognized the formation of five clades, including *Rubia*, *Sherardia* L., *Asperula* L. sect. *Glabella* clade, *Cruciata* Mill., and *Galium* L. sect. *Galium*. This analysis placed species of *Galium* in three of the five clades established. Manen et al. (1994) and Bremer and Manen (2000) viewed the genus as of probable paraphyletic origin, while Manen and Natali (1995) considered *Galium* polyphyletic. These differences of opinion with relation to the origin of these taxa are to be expected, as the genus comprises more than 400 species. Bremer and Manen (2000) defined *Relbunium* as a monophyletic group among the Rubieae, which agrees with the morphological data presented here.

Dempster prepared an ample revision of *Galium* species from North, South, and Central America (Dempster & Ehrendorfer, 1965; Dempster, 1968, 1970, 1975, 1978, 1980, 1981, 1982, 1990). For the South American taxa, Dempster (1982, 1990) discussed the validity of *Relbunium* as a genus and considered it to be a section of the genus *Galium*. Endlicher (1839: 523) proposed the name *Relbunium* as a section of *Galium*, based on de Candolle (1839) for *Rubia* sect. *Involucratae*, and described it as: "...Pedunculi axillares apice gerentes bracteas quatuor verticillatas, involucrum constituentes, flores intra involucrum solitarii v. terni, sessiles v. pedicellati." Dempster (1982) observed that this definition allowed the generic inclusion of not only sessile involucrate flowers, but also involucrate inflorescences. This author affirmed that Endlicher's reference to pedicellate flowers actually referred to branchlets instead of true pedicels. It is worth noting that Dempster did not refer to the pedicels of the flowers of *Relbunium* as true pedicels, since the presence of bracts obliged this author to define them as false pedicels. It may be inferred that Dempster assumed Endlicher permitted the inclusion of flowers with true pedicels (those without bracts) in *Relbunium*. Therefore, it is easy to understand why Dempster mentioned that Endlicher did not refer to the pedicels with bracts as being true pedicels. However, in the description presented by Endlicher (1839), one cannot perceive any mention of pedicellate flowers without bracts, a condition defined by Dempster as being critical in the decision to validate *Relbunium*.

When later elevating *Relbunium* to a genus, Bentham and Hooker (1873: 149) described it as: "...Herbae habitu follis floribusque Galli, flores bracteis 4 involucrati, sed inflorescentia involucrata, fructuque saepissime carnoso, pedicello cum calyce articulato..." In agreement with Dempster (1982), these authors continued to accept the permissive definition of Endlicher and to include flowers without bracts into *Relbunium*. Ehrendorfer (1955: 518) provided a more detailed description without altering the significance of the previous proposals: "...Flores sessiles vel rarius brevissime pedicellati, bracteis quaternis vel binis involucrati; bracteae involucrales nonnunquam ipsae pedunculos gerentes." Dempster (1982: 212–213) affirmed that, in using the word "peduncles" in this description, Ehrendorfer was emphasizing that these species had branchlets with true pedicels. However, the presence of bracts is sufficient evidence to maintain *Relbunium* as genus in this paper, given the relevance of this character in defining the group.

In contrast, Dempster (1982) determined that the flowers in *Relbunium* are individually involucrate and/or sessile. As such, this author transferred the species of *Relbunium* that did not have involucrate inflorescences (*R. richardianum*, *R. equisetoides*, and *R. diphyllum* K. Schum.) to *Galium*, affirming these species as similar to *G. suffruticosum*, *G. araucanum* (Dempster, 1982), *G. microphyllum* A. Gray, and *G. corymbosum* Ruiz & Pav. (Dempster, 1990). Dempster (1982) observed such variation among these species of *Galium*, such as occasional bracts at the base of the inflorescences, thus giving rise to the *G. richardianum* (Gillies ex Hook. & Arn.) Endl. complex (Dempster, 1982). This *G. richardianum* complex fills a gap that exists between the genera *Relbunium* and *Galium*, as all of the individuals of *G. suffruticosum* have flowers with pedicels, while the otherwise similar species *G. araucanum* has sessile flowers, as seen in some *Relbunium* species. However, *G. araucanum* (like *G. richardianum*) has few flowers with bracts. There are many similarities between *G. araucanum* and *G. richardianum*, and these two species differ principally in terms of the surfaces of their fruits, which are tuberculate in *G. richardianum* and smooth in *G. araucanum*. Thus, involucrate, sessile, and solitary flowers probably originated from the primitive pedicellate and cymose condition in *Galium* (Dempster, 1982). In 1990, Dempster transferred ca. 12 species of *Relbunium*, including them in *Galium*.

The generic segregation of *Relbunium* from *Galium* has been confirmed by the work of Detoni (1976) and Cavalli (1976), who used analyses of flavonoids and isozymes and indicated that *R. hypocarpium* was the most basal species of its genus at that time. The current morphological evidence confirms the segregation of *Relbunium* from *Galium* and maintains *Relbunium* as an autonomous genus. However, the basal position of *R. hypocarpium* within the genus could not be confirmed by the morphological characteristics of the reproductive organs, because that position is occupied by *R. humile* and *R. mazocarpum*.

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APPENDIX 1. Morphological characters and character states in *Galium* and *Relbunium*. Compare Appendix 2 and Table 1 for species exemplars.

1. Flower: (0) bisporic, (1) monosporic.
2. Inflorescences: (0) absent, (1) present, (2) both inflorescences and solitary flowers present.
3. Inflorescence morphology: (0) monochasium, (1) dichasium, (2) panicle, (3) pseudanthia.
4. Peduncle: (0) absent, (1) present.
5. Peduncle: (0) to 5 mm (short), (1) 5–10 mm (medium), (2) 10–15 mm (long), (3) to 20 mm (longer).
6. Floral peduncle: (0) erect, (1) curved.
7. Floral bracts: (0) absent, (1) present.
8. Bract number: (0) 2, (1) 2 or 3, (2) 4.
9. Bract presentation: (0) equal, (1) unequal.
10. Bract shape: (0) wider than long, (1) longer than wide.
11. Bract position: (0) not enveloping flowers and fruits, (1) enveloping flowers and fruits.
12. Reduced pedicel: (0) absent, (1) present.
13. Reduced pedicel: (0) imperceptible, (1) less than 0.7 mm, (2) 0.7–1.1 mm.
14. Calyx: (0) evident, (1) reduced, (2) absent.
15. Corolla shape: (0) campanulate, (1) infundibular, (2) rotate.
16. Corolla epidermal cuticle surface: (0) wrinkled, (1) striate.
17. Corolla abaxial epidermis, unicellular trichomes: (0) absent, (1) marginal, (2) dispersed.
18. Corolla adaxial epidermis, trichomes: (0) unicellular, (1) glandular.
19. Corolla, glandular trichomes: (0) dispersed, (1) marginal and along central vein.
20. Corolla, marginal glandular trichomes in: (0) 1 row, (1) 2 or 3 rows, (2) 3 to 5 rows.
21. Corolla, glandular trichomes along the central vein in: (0) 1 row, (1) 2 or 3 rows.
22. Corolla abaxial epidermis, idioblasts: (0) absent, (1) present.
23. Corolla mesophyll: (0) 2 to 4 layers, (1) 4 to 6 layers, (2) more than 6 layers.
24. Corolla mesophyll, intercellular spaces: (0) reduced, (1) abundant.
25. Corolla mesophyll, crystalliferous idioblasts: (0) next to adaxial epidermis, (1) in the mesophyll.
26. Ovary, periclinal external wall of epidermal cells: (0) plain, (1) rounded.
27. Ovary, cuticle of the epidermal cells: (0) wrinkles, (1) striae.
28. Ovary, striae orientation: (0) random, (1) parallel, (2) into the cell center.
29. Ovary: (0) glabrous, (1) pubescent.
30. Ovary, with idioblasts: (0) absent, (1) present.
31. Ovary, rounded appearance to epidermal cells: (0) absent, (1) present.
32. Fruits with the reduced pedicel: (0) not elongating, as in the flowers, (1) elongating in fruit, longer than in flowers.
33. Fruits: (0) fleshy, (1) dry.
34. Fruits fleshy and as: (0) berry, (1) drupe.
35. Fruits dry and as: (0) septicidal capsules, (1) schizocarp.
36. Exocarp: (0) glabrous, (1) pubescent.
37. Fruits with the idioblasts: (0) absent, (1) present.
38. Exocarp cells with periclinal divisions: (0) absent, (1) present.

39. Exocarp with papillae: (0) absent, (1) present.
40. Exocarp idioblasts with: (0) striae short cuticle, (1) striae long cuticle.
41. Exocarp idioblasts: (0) isolated, (1) isolated and in groups.
42. Mesocarp cells: (0) isodiametric, (1) elongated.
43. Mesocarp, cellular layers in early fruits: (0) 3 to 5, (1) 4 to 7, (2) more than 7.
44. Mesocarp, cellular layers in mature fruits: (0) 2 to 3, (1) 4 to 6, (2) 7 to 10, (3) more than 10.
45. Mesocarp, periclinal divisions: (0) absent, (1) present.
46. Mesocarp, cells: (0) isodiametrics, (1) as palisade tissue.
47. Mesocarp, lacunae: (0) absent, (1) present.
48. Mesocarp, intercellular spaces: (0) not frequently, (1) very frequently.
49. Mesocarp, succulent: (0) absent, (1) present.
50. Endocarp, single layer: (0) absent, (1) present.
51. Endocarp: (0) discontinuous, (1) continuous.
52. Endocarp entire: (0) visible, (1) invisible.
53. Seed coat: (0) single layer of cells, (1) more than one layer.
54. Seed coat, cellular layer: (0) entire, (1) fragmented.
55. Seed coat, cells: (0) without tannin drops, (1) with tannin drops.

APPENDIX 2. Morphological matrix. In addition to numerical character states, polymorphic characters are represented as ? = unknown state, – = inapplicable.

	Characters																																																						
Species	1									10										20										30										40										50					55
Galium latoramosum	1	2	1	1	0	0	0	–	–	–	–	0	–	2	2	0	0	1	1	2	1	0	0	0	0	0	1	1	0	0	0	–	1	–	1	0	0	1	0	–	–	0	1	2	1	0	1	0	1	1	0	–	0	0	0
G. uruguayense	0	0	–	1	0	0	0	–	–	–	–	0	–	2	2	0	2	1	0	–	–	0	0	0	0	0	1	1	0	1	0	–	1	–	1	0	1	0	0	0	0	1	1	0	0	0	1	1	0	1	1	0	0	0	0
Relbunium equisetoides	0	1	1	1	0	0	1	2	1	1	0	1	0	2	2	0	0	1	1	1	0	0	0	1	0	0	0	–	0	0	0	0	1	–	1	0	0	0	0	–	–	1	0	0	0	1	1	1	0	1	1	1	0	0	0
R. gracillimum	0	0	–	1	2	0	1	2	0	1	0	1	1	2	2	?	0	1	1	?	?	1	0	0	0	1	0	–	0	1	1	0	0	0	–	0	0	0	0	–	–	0	0	0	0	0	1	0	1	1	1	0	0	0	0
R. hirtum	0	0	–	0	–	–	1	2	0	1	0	1	0	2	2	1	2	1	1	0	0	0	1	0	0	1	0	–	0	0	1	0	1	–	1	0	0	0	1	–	–	1	0	0	0	0	1	0	0	1	0	–	0	0	1
R. humile	0	0	–	1	0	0	1	0	0	1	0	1	2	2	2	0	1	1	1	1	0	0	0	1	0	0	1	1	1	0	0	1	1	–	1	1	0	0	0	–	–	1	0	0	0	0	1	0	0	1	0	–	0	0	0
R. humilioides	0	0	–	1	1	0	1	2	0	0	1	1	1	2	2	0	2	1	1	1	0	0	1	1	1	1	1	0	0	0	1	0	1	–	1	0	0	0	0	–	–	1	0	0	0	0	1	1	0	1	1	0	0	0	0
R. hypocarpium	0	0	–	1	2	0	1	2	0	0	0	1	0	2	2	0	1	1	1	1	1	0	0	1	0	0	0	–	1	0	0	0	0	0	–	1	0	0	0	–	–	0	0	0	0	0	1	0	1	1	1	0	0	1	0
R. longipedunculatum	0	0	–	1	2	1	1	2	1	1	0	1	1	2	2	0	0	1	1	2	1	0	1	0	0	1	1	1	0	0	1	0	1	–	1	0	0	0	1	–	–	1	0	2	0	0	1	0	0	1	1	0	0	0	1
R. mazocarpum	0	0	–	1	0	1	1	0	0	1	0	1	2	2	2	0	1	1	1	1	0	0	0	1	0	0	1	1	0	1	0	1	1	–	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	1	0	–	0	0	0
R. megapotamicum	0	0	–	1	0	0	1	2	0	1	0	1	0	2	2	0	0	1	1	1	0	0	1	0	0	0	1	2	0	1	1	0	1	–	1	0	1	0	1	1	1	1	0	0	0	0	1	1	0	1	1	0	0	0	0
R. nigro-ramosum	0	0	–	1	0	0	1	2	1	1	0	1	0	2	2	1	0	1	1	1	0	0	1	0	0	1	1	2	0	1	1	0	1	–	1	0	1	0	0	1	1	1	1	1	0	0	1	1	0	1	1	1	0	0	0
R. ostenianum	0	0	–	0	–	–	1	1	0	1	0	1	0	2	2	0	2	1	1	1	1	0	1	0	1	0	1	2	1	0	1	0	1	–	1	1	0	0	1	–	–	1	1	1	0	0	1	0	0	1	1	1	0	0	1
R. richardianum	0	2	0	1	0	0	1	2	0	1	0	1	0	2	2	0	0	1	1	1	0	0	1	0	0	0	1	0	0	1	1	0	1	–	1	0	1	0	1	0	0	1	1	0	0	0	1	0	0	1	0	–	0	0	0
R. valantioides	0	0	–	1	1	0	1	2	0	0	1	1	1	2	2	0	1	1	1	1	0	0	1	0	0	0	1	1	0	0	0	0	1	–	1	0	0	0	0	–	–	1	0	0	0	0	1	0	0	1	1	0	0	0	0
Borreria verticillata	0	1	3	0	–	–	1	2	0	1	0	0	–	0	1	?	2	?	?	?	?	0	2	1	0	0	?	?	1	0	0	–	1	–	0	1	0	0	0	–	–	1	2	1	0	0	0	0	0	0	1	0	0	0	1
Psychotria carthagenensis	0	1	2	1	3	0	1	0	0	1	0	0	–	1	0	?	2	?	?	?	?	0	2	1	0	0	?	?	0	0	0	–	0	1	–	0	0	0	0	–	–	0	2	3	1	0	0	0	1	0	1	0	1	0	0